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# Quantitation of Two Endogenous Lactose-inhibitable Lectins in Embryonic and Adult Chicken Tissues

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**ABSTRACT** Two lactose-binding lectins from chicken tissues, chicken-lactose-lectin-I (CLL-I) and chicken-lactose-lectin-II (CLL-II) were quantified with a radioimmunoassay in extracts of a number of developing and adult chicken tissues. Both lectins could be measured in the same extract without separation, because they showed no significant immunological cross-reactivity. Many embryonic and adult tissues, including brain, heart, intestine, kidney, liver, lung, muscle, pancreas, and spleen, contained one or both lectins, although their concentrations differed markedly. For example, embryonic muscle, the richest source of CLL-I contained only traces of CLL-II whereas embryonic kidney, a very rich source of CLL-II contained substantial CLL-I. In both muscle and kidney, lectin levels in adulthood were much lower than in the embryonic state. In contrast, CLL-I in liver and CLL-II in intestine were 10-fold to 30-fold more concentrated in the adult than in the 15-d embryo. CLL-I and CLL-II from several tissues were purified by affinity chromatography and their identity in the various tissues was confirmed by polyacrylamide gel electrophoresis, isoelectric focusing, and peptide mapping. The results suggest that these lectins might have different functions in the many developing and adult tissues in which they are found.

Lectins are divalent or polyvalent carbohydrate-binding proteins that can be assayed as agglutinins. Although they are widely distributed in nature, little is presently known about their function (1). Chicken tissues contain at least four lectins (3, 6, 7, 10, 14), two of which react with lactose and other  $\beta$ -galactosides (3). One, designated chicken-lactose-lectin-I (CLL-I) has been purified from both embryonic muscle and adult liver (3, 7, 14) and appears to be identical in both these tissues by a number of criteria (3). A second lactose-inhibitable lectin from chicken tissues, chicken-lactose-lectin-II (CLL-II) has recently been purified from adult chicken intestine and shown to be distinct from CLL-I in that it has a smaller subunit molecular weight, is isolated largely as a monomer rather than a dimer, and has a strikingly different isoelectric point and peptide map. However, CLL-I and CLL-II have similar carbohydrate binding sites in that both are adsorbed by an asialofetuin-Sepharose affinity column and the hemagglutination activity of both is inhibited by the same saccharides (3). Therefore, it is not possible to quantify the concentrations of each of these lectins in tissue extracts by hemagglutination tests.

In the present report we show that CLL-I and CLL-II can be readily distinguished and individually measured by radioimmunoassays, because antisera raised against them show no

crossreaction over a concentration range of at least three orders of magnitude. Using the radioimmunoassays we were able to quantify CLL-I and CLL-II in extracts of many chicken tissues. We found that many tissues contained both lectins, but in very different proportions. Furthermore, the proportions and levels showed some marked changes with tissue differentiation and maturation. For example CLL-I is concentrated in embryonic muscle and adult liver whereas CLL-II is concentrated in embryonic kidney and adult intestine. In some tissues we confirmed the results of the radioimmunoassays by purifying both lectins and directly demonstrating their relative amounts. This ruled out the possibility that the radioimmunoassays were measuring other cross-reactive materials. The corresponding lectins in all tissues showed no apparent differences, and all purified samples of CLL-I had peptide maps that were indistinguishable, as did all purified samples of CLL-II.

## MATERIALS AND METHODS

### *Lectin Extraction and Purification*

Lectins were extracted from embryonic and adult chicken tissues by homogenization in 9 vol of 75 mM NaCl, 75 mM  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ , pH 7.2, containing 4 mM  $\beta$ -mercaptoethanol, 2 mM EDTA, and 300 mM lactose, followed by centrifugation at 100,000 g for 1 h, as described previously (14). Protein concen-

trations of crude extracts and purified lectins were determined by the method of Bradford (5).

Lectins were purified by affinity chromatography on asialofetuin-derivatized Sepharose as described previously (2, 3). The lectins were specifically eluted from the affinity column with 0.3 M lactose. In some experiments, further purification was achieved by preparative isoelectric focusing performed for 4 d at 4°C in an LKB isoelectric focusing apparatus (LKB Instruments, Inc., Rockville, Md.) as described previously (3). Polyacrylamide gel electrophoresis in SDS using appropriate molecular weight markers, and mapping of  $^{125}\text{I}$ -labeled tryptic peptides prepared after iodinating protein bands from polyacrylamide-SDS gels, were also done as described previously (3).

## Antibody Preparation and Characterization

Rabbit antiserum that had been raised against CLL-I purified from adult liver has been characterized previously (2, 3). Rabbit antiserum was raised against CLL-II that had been purified from adult intestine by affinity chromatography and isoelectric focusing. Rabbits were immunized by intradermal injection of 100  $\mu\text{g}$  of purified lectin in Freund's complete adjuvant followed 3 wk later by an intradermal booster injection of 50  $\mu\text{g}$  of antigen in incomplete Freund's adjuvant and 3 wk thereafter by an intravenous injection of 10  $\mu\text{g}$  of antigen in saline. Sera were harvested 7 d later.

Ouchterlony double immunodiffusion was conducted as described previously in agarose containing 50 mM lactose to prevent interaction of the lectins with the agarose or serum glycoproteins (3). Immunoelectrophoresis was performed in 1.2% agarose containing 0.05 M barbital buffer, pH 8.4 and 50 mM lactose. Electrophoresis was conducted at 10°C at a constant voltage of 100 V/plate using an LKB Multiphor apparatus. After electrophoresis, antibody was added to the troughs and gels were incubated overnight in a moist chamber. Gels were washed extensively, dried, and stained with Amido black.

## Radioimmunoassay

Purified CLL-I prepared from adult liver (2, 3) and CLL-II prepared from adult intestine (3) were radioiodinated with  $^{125}\text{I}$  by a slight modification of the method of Bolton and Hunter (4). Affinity purified lectin was dialyzed overnight against 3,000 volumes of 0.1 M sodium borate, pH 8.5 at 4°C. 1 mCi of  $^{125}\text{I}$ -labeled Bolton-Hunter reagent (Amersham-Searle, Arlington Heights, Ill.) in benzene was brought to dryness under a gentle  $\text{N}_2$  stream at 4°C and 15  $\mu\text{g}$  of lectin in 30  $\mu\text{l}$  was delivered to the bottom of the vial. The solution was mixed vigorously for 2–3 min at 4°C, incubated on ice for 30 min, then 0.5 ml of 200 mM glycine in 0.1 M sodium borate pH 8.5 was added and the mixture was incubated a further 5 min on ice. This reaction mixture was applied to a 10 ml Sephadex G-25 column that had been previously equilibrated with phosphate-buffered saline pH 7.2, containing 0.25% gelatin and 30 mM lactose and was eluted with that same buffer. The void volume containing  $^{125}\text{I}$ -lectin was collected and pooled.  $^{125}\text{I}$ -lectins had an average specific activity of  $\sim 20 \mu\text{Ci}/\mu\text{g}$ . At least 95% of the iodinated lectin was precipitable with cold 10% trichloroacetic acid or in the radioimmunoassay.

The radioimmunoassay samples and reagents were prepared or diluted into 75 mM NaCl, 75 mM  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ , pH 7.2 containing 50 mM galactose and 2 mg/ml bovine serum albumin. Assay mixtures were prepared by sequential addition of: (a) 100  $\mu\text{l}$  of either the sample to be assayed or standard unlabeled antigen; (b) 25  $\mu\text{l}$  of an appropriate dilution of antilectin antiserum (typical concentrations: anti CLL-I, 1/6,000; anti CLL-II, 1/600) containing a 1/300 dilution of normal rabbit serum; (c) 25  $\mu\text{l}$   $^{125}\text{I}$ -lectin, containing  $\sim 20,000$  cpm. This mixture was incubated for 20 min at room temperature. Then, 50  $\mu\text{l}$  of a 10% suspension of formaldehyde-fixed whole *Staphylococcus aureus* (IgG-sorb, New England Enzyme Center, Boston, Mass.) was added and the mixture was incubated for a further 15 min on ice. After addition of 2 ml buffer the tubes were centrifuged at 700 g for 10 min. Supernatants were carefully poured off and radioactivity in the pellets was determined with a gamma counter. The assay conditions were arranged so that in the absence of competing antigen  $\sim 50\%$  of the radioactive antigen was precipitated. The competing antigen was screened at a number of concentrations and accurately measured in triplicate at concentrations within the linear range of the assay, as determined with unlabeled pure antigen. The number of counts precipitated in the absence of immune serum, up to 5% of the total, was taken as background and subtracted from all determinations.

## RESULTS

### Antibody Characterization

Antiserum raised against CLL-II that had been purified from adult chicken intestine reacted strongly with purified antigen (Fig. 1). Soluble extracts of chicken intestine contained

no other immunologically crossreactive material as measured by immunoelectrophoresis (Fig. 1). Other evidence that the antisera against both CLL-I and CLL-II react only with these materials in the tissue extracts will be presented below. Other characteristics of the anti-CLL-I serum have already been described (2, 3).

### Specificity of Radioimmunoassays

Highly sensitive radioimmunoassays were developed for CLL-I and CLL-II. The assay for CLL-I could measure  $<0.1$  ng of antigen whereas the assay for CLL-II was slightly less sensitive (Fig. 2). Both assays were linear over an antigen concentration range exceeding one order of magnitude. No immunological crossreactivity between CLL-I and CLL-II was found with the radioimmunoassays even when very large amounts of the potentially crossreactive material were used (Fig. 2). Therefore, small amounts of one lectin could be reliably detected in extracts rich in the other lectin.

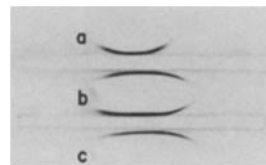


FIGURE 1 Immunoelectrophoretic analysis of antiserum raised against CLL-II purified from adult intestine. Immunoelectrophoresis was conducted as described in Materials and Methods. Troughs contained 100  $\mu\text{l}$  of anti-CLL-II serum. Well contents were: a) 2  $\mu\text{g}$  purified CLL-II; b) 10  $\mu\text{l}$  crude intestinal extract; c) 2  $\mu\text{g}$  purified CLL-II and 10  $\mu\text{l}$  crude intestinal extract.

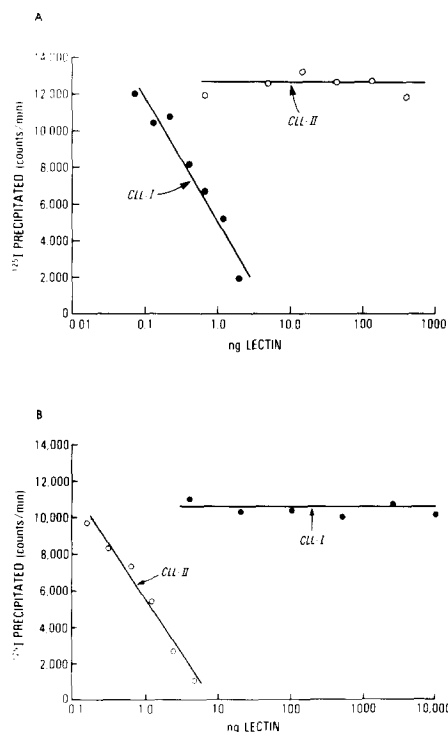


FIGURE 2 Radioimmunoassays. Procedural details are described in Materials and Methods. a) Assay for CLL-I, using anti CLL-I serum and  $^{125}\text{I}$ -CLL-I probe. b) Assay for CLL-II, using anti CLL-II serum and  $^{125}\text{I}$ -CLL-II probe. Unlabeled CLL-I (●) and CLL-II (○) were added in the amounts indicated.

TABLE I  
Lectin Concentration in Extracts of Embryonic and Adult Chicken Tissues

Tissue	Lectin concentration (ng lectin/mg protein)			
	CLL-I	CLL-II	CLL-I	CLL-II
	15-d embryo		Adult	
Liver	130 ± 20	250 ± 60	1,000 ± 80	23 ± 7
Intestine	160 ± 30	600 ± 100	80 ± 27	18,000 ± 4,000
Pectoral muscle	3,600 ± 200	≤2	9 ± 1	3 ± 2
Thigh muscle	3,800 ± 300	≤3	13 ± 2	6 ± 2
Pancreas	630 ± 30	550 ± 100	950 ± 100	80 ± 5
Kidney	720 ± 200	5,600 ± 1,700	400 ± 40	360 ± 23
Heart	190 ± 60	≤2	200 ± 40	6 ± 2
Brain	240 ± 80	5 ± 2	520 ± 100	6 ± 2
Spleen	*	*	50 ± 20	30 ± 10
Gizzard	*	*	240 ± 60	3 ± 1
Lung	*	*	23 ± 2	4 ± 2

Tissues were extracted as described in Materials and Methods. The soluble extracts were assayed for lectins by radioimmunoassay and for protein concentration. Values shown represent the mean ± SEM of at least three separate determinations.

\* Not tested.

### Lectin Concentration in Developing Tissues

Extracts of many embryonic and adult chicken tissues contain substantial amounts of CLL-I and CLL-II (Table I). Both lectins showed striking changes in concentration at different stages in the development of some tissues (Table I, Fig. 3). As already inferred from hemagglutination assays (14), CLL-I changed strikingly with the development of pectoral muscle, rising to a peak at around 12 d of embryonic development and falling to very low levels in the adult. Whereas CLL-II was present at very low levels in both embryonic and adult muscle, it was abundant in embryonic kidney but fell to low levels in that adult tissue as well. In contrast, CLL-II levels in intestinal extracts rose strikingly in adulthood. The results presented in Table I and Fig. 3 indicate that although both CLL-I and CLL-II are developmentally regulated proteins in some tissues, neither is always characteristic of either the embryonic or the adult state. Actively developing tissues and adult tissues may be rich in one or both.

We also determined the concentration of both lectins in whole 4-d old chicken embryos. Extracts contained 150 ng CLL-I and 320 ng CLL-II per milligram of soluble protein. Therefore, these lectins are already prominent proteins in very early embryos, although much higher levels are found in some developing or adult tissues.

### Lectin Purification

To evaluate the possibility that we were measuring immunologically crossreactive soluble material in these extracts, we purified the lectins from three of the richest tissue sources, adult kidney, adult pancreas, and embryonic liver. We applied extracts of these tissues to affinity columns and eluted the lectins with lactose. We found that all the immunologically reactive material bound to the affinity column and was eluted with lactose. By this criterion all of the immunologically reactive material was, indeed, lectin.

We then examined the purity of these preparations eluted from the affinity column by polyacrylamide gel electrophoresis and compared them with pure CLL-I from adult liver and pure CLL-II from adult intestine (Fig. 4). The material from adult pancreas gave a single band which electrophoresed like CLL-I (Fig. 4). The small amount of CLL-II detected by the radioim-

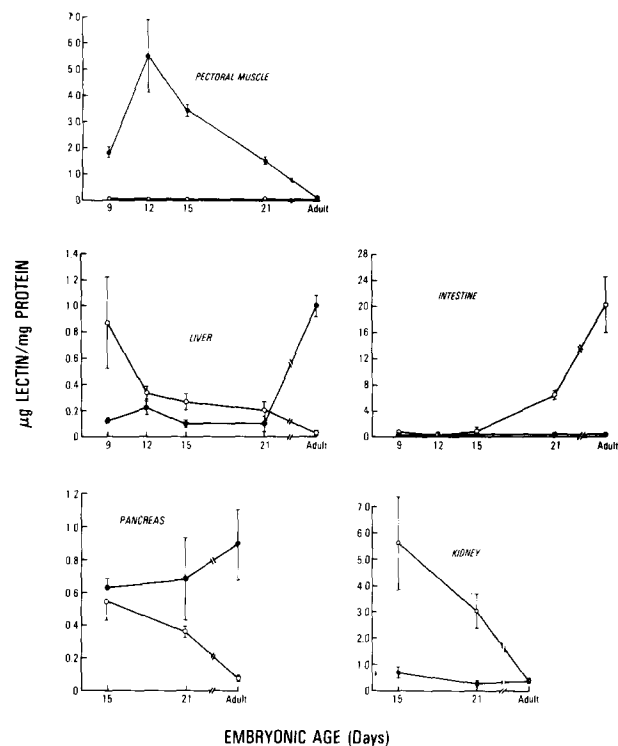


FIGURE 3 Concentration of CLL-I (●) and CLL-II (○) in soluble extracts of chicken tissues at various stages of development. Lectins were assayed by radioimmunoassay. Each point is the mean (+/- SEM) of results with three to five different preparations.

munoassay was apparently below the limits of detection of the polyacrylamide gel. In contrast, the purified materials from both embryonic liver and adult kidney showed two bands that migrated like CLL-I and CLL-II (Fig. 4), as would be expected from the radioimmunoassay studies.

Although comigration on polyacrylamide gels suggested the identity of all the material with identical subunit molecular weights, we evaluated this further in a number of ways. Because CLL-I and CLL-II have characteristic isoelectric points, we subjected the purified material from adult kidney to preparative isoelectric focusing. We found that CLL-I immunoreactive material was concentrated in a single peak at pH 4.0 and CLL-

II was concentrated at a single peak at pH 6.2. These isoelectric points are indistinguishable from those previously determined for purified materials from muscle, liver and intestine (3, 14). We also examined the affinity-purified preparations by immunodiffusion. All the materials that reacted with antisera raised against CLL-I or CLL-II showed precipitin lines that merged without spurring indicating that they were indistinguishable by this criterion. As a more stringent test for identity we prepared tryptic peptide maps of  $^{125}\text{I}$ -labeled peptides obtained by iodination of single bands from SDS polyacrylamide gels. We found that the peptide maps of CLL-I from adult liver, kidney, pancreas, and embryonic liver did not differ significantly (Fig. 5). The slight differences observed were no greater than those found when duplicate maps were prepared from the same sample. Similarly, CLL-II from adult intestine and kidney and embryonic liver showed peptide maps that were indistinguishable (Fig. 5).

## DISCUSSION

Previous studies have identified two distinct lactose-inhibitable lectins, CLL-I and CLL-II, in chicken tissues (3) and have shown that the level of lactose-sensitive hemagglutination activity changes with development of some tissues (8, 11, 13). However, the two lectins responsible for this hemagglutination activity could not be individually measured in tissue extracts by the hemagglutination assay. In the present study we describe radioimmunoassays that permit separate measurement of CLL-I and CLL-II in tissue extracts. With these assays we show that levels of CLL-I and CLL-II show striking changes with development of certain tissues. For example, CLL-I is abundant in developing muscle and virtually undetectable in the adult. Yet this same lectin becomes very abundant in adult liver. In contrast the CLL-II levels in adult liver are much lower than those of the embryo. In kidney CLL-II is also much more abundant in the embryo than in the adult, whereas CLL-I shows relatively little change with development; but, like CLL-I, CLL-II is not always characteristic of the embryonic state because its levels in intestine only become prominent late in development.

Conclusions from these studies were confirmed by purifying the lectins from a number of tissues by affinity chromatography. The purified samples of CLL-I or CLL-II from different tissues were indistinguishable when tested by polyacrylamide gel electrophoresis, isoelectric focusing, immunodiffusion, or peptide mapping. Therefore, the results suggest that two iden-

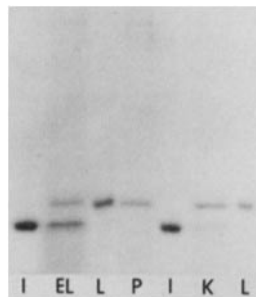


FIGURE 4 Polyacrylamide gel electrophoresis in SDS of lectins purified by affinity chromatography from embryonic and adult chicken tissues. Tissue sources of purified lectins were: I, adult intestine; EL, 14-d embryonic liver; L, adult liver; P, adult pancreas; K, adult kidney.

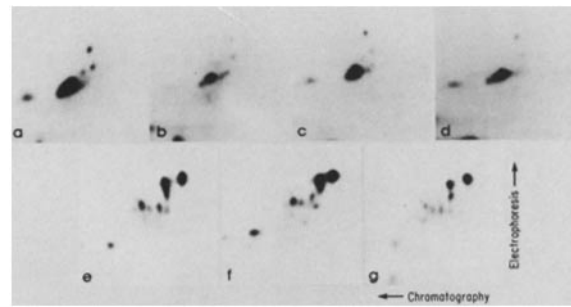


FIGURE 5  $^{125}\text{I}$ -labeled tryptic peptide maps of purified lectins. Affinity purified lectins were electrophoresed in polyacrylamide gels in SDS as shown in Fig. 4. Bands that had the same molecular weight as CLL-I from liver or CLL-II from intestine were cut out of the gels, iodinated, digested with trypsin and mapped. The maps were all prepared under identical conditions. The arrows indicate the directions of chromatography and electrophoresis. The origin was located at the lower right hand corner of the maps. The proteins studied were: CLL-I from (a) adult liver, (b) embryonic liver, (c) adult pancreas, (d) adult kidney; and CLL-II from (e) adult intestine, (f) embryonic liver, and (g) adult kidney.

tical lectins that are found in many tissues undergo developmentally regulated tissue-specific changes in levels.

There is considerable evidence that synthesis of lectins accompanies the differentiation of cellular slime molds from a vegetative to an aggregating state and that the lectins participate in cell-cell adhesion (1). The developmental role of CLL-I and CLL-II is difficult to interpret from the available evidence. In the case of CLL-I some studies support its participation in myoblast interactions that culminate in cell fusion (9, 12, 15). However, the lectin is clearly not confined to this function, because it is abundant in embryonic and adult tissues where fusion is not a prominent event. Studies of the localization of CLL-I in adult tissues (2) suggest that it may have multiple functions that have not been clearly defined.

As with many other lectins (1) nothing is yet known about the function of CLL-II in either embryonic or adult tissues. It is anticipated that the potent and specific antibodies directed against CLL-I and CLL-II described here will facilitate an analysis of the endogenous functions of these lectins. Use of these antibodies to evaluate the function of CLL-I and CLL-II in adult intestine will be described in the following report.

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